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(54) Title: METHODS FOR INDUCING CHONDROGENESIS AND PRODUCING *DE NOVO* CARTILAGE *IN VITRO*

(57) Abstract: Methods of producing cartilage *in vitro* are described. Also described are methods of promoting chondrocytic differentiation, methods of enhancing chondrogenesis, as well as methods of producing implantable cartilaginous cultures. The methods feature, in particular, the culturing of pleuripotent mesenchymal stem cells. The methods are particularly useful in the repair of cartilage as well as augmentation of cartilage *in vivo*, for example, in a patient in need of cartilage repair and/or cartilage augmentation. Also featured are implantable cartilaginous cultures.

METHODS FOR INDUCING CHONDROGENESIS AND PRODUCING *DE NOVO* CARTILAGE *IN VITRO*

Background of the Invention

Cartilage is a hypocellular tissue consisting of extracellular matrix molecules, water, and a relatively small population of chondrocytes. Caltilage provides elasticity at the junction of bones of the mammalian skeletature (*e.g.*, among the bones of the rib cage), provides structure to superficial organs such as the nose and ears, and plays a critical role in absorbing shock and minimizing stress on the bones. There are at least three prominent types of cartilage including elastic cartilage, fibrocartilage, and articular or hyaline cartilage, the latter being archetypic for the appendicular skeleton and the vertebral column. Hyaline cartilage arises from pluripotent mesenchymal ancestor cells that remain morphologically undifferentiated prior to a localized cell condensation in specific regions destined to undergo chondrogenesis. Injuries to articular cartilage can result in numerous clinical symptoms, such as pain and decreased functional levels. The limited reparative capabilities of hyaline cartilage often results in the generation of repair tissue that lacks the structure and biomechanical properties of normal cartilage. In response to blunt, superficial, or deep penetrating trauma, for example, chondrocytes are unable to adequately proliferate, migrate, and synthesize high-quality repair tissue.

Therapies for the treatment of articular cartilage injuries or degradation rely in great part on the ability to culture chondrocytes in culture. At present, the available treatments depend on the isolation of chondrocytes from one area of a patient and autologous implantation of those cells at the site of injury. Various permeations of this technique involve culturing the chondrocytes in collagen gels or matrices to provide mechanical strength. Alternatively, entire autologous cartilage grafts can be used, for example, in reconstruction procedures. A significant drawback of these procedures, however, is the paucity of the graftable material. Accordingly, there exists a need for methods of synthesizing *de novo* cartilage from cultured cells for implantation and reconstruction.

Systems previously described for the cultivation of cartilage cells include both monolayer or organoid (high density) cultures. In monolayer culture, isolated chondrocytes are grown at low density in serum-containing media, however, after a short culture period, the cells dedifferentiate, are fragmented to fibroblast-like cells (Grundmann, *et al.*, 1980; Quarto *et al.*, 1990; Shakibaei, 1995; Shakibaei *et al.*, 1996), form collagen type I (Benya *et al.*, 1977; von der Mark *et al.*, 1977; von der Mark, 1980), change the pattern of their proteoglycan synthesis (Kim and Conrad, 1977; Benya and Shaffer, 1982) and that of their surface receptors (Shakibaei, 1995), or they are overgrown by fibroblast-like cells. Longer periods of culture are needed for the observation of certain aspects of chondrogenesis (*e.g.*, maturation or mineralization).

In organoid or high-density cultures chondrocytes are either transformed to fibroblast-like cells after a 2- to 3- week culture period (Norby *et al.*, 1977; Schröter-Kermani *et al.*, 1991; Shakibaei *et al.*, 1993a, b) or develop to hypertrophic cartilage cells whose matrix can mineralize (Osdoby and Caplan, 1979; Tacchetti *et al.*, 1987; Gerstenfeld and Landis, 1991; Zimmermann *et al.*, 1992). Another disadvantage of these methods is that they do not yield pure cartilage cultures but cultures with a perichondrium and unspecific connective tissue between the cartilage nodules (Zimmermann *et al.*, 1992; Shakibaei *et al.*, 1993a; b; 1995).

Attempts have also been made to generate chondrocyte cultures from pleuripotent mesenchymal cells. For example, the murine mesenchymal 10T1/2 cells is a pleuripotent cell line capable of differentiating originally into the three distinct lineages of muscle, fat, and cartilage upon 5-azacytidine addition (Taylor and James, 1979). Cartilage cells account for about 0.1-1% of the total number of differentiated cells, with the majority of cells (25-50%) differentiating into the muscle lineage. Similarly, confluent monolayers of 10T1/2 fibroblasts stably transfected with BMP-2 or BMP-4 differentiate into the three lineages of bone, fat and cartilage (Ahrens *et al.*, 1993). However, only 0.2% of the BMP-2 expressing cells differentiate to these lineages and, of those, chondrogenic cells are represented at 5 to 25-fold fewer cells than cells of the adipogenic lineage. Exogenous addition of BMP-2 to confluent cultures of 10T1/2 cells produces a similar effect as stable BMP-2 transfection (Katagiri *et al.*, 1990; Wang *et al.*, 1993). Similar results have likewise been obtained with other

pleuripotent cell lines and cell lines and cells isolated from the periosteum. To date, no success has been reported in inducing chondrogenesis *in vitro* using primary cells.

The evidence obtained using 10T1/2 cells and other cells suggests that both the cellular and growth factor environment are important for cartilage fate determination *in vitro*. However, while these systems (*e.g.*, the 10T1/2 micromass system) can be employed as a model for understanding chondrogenesis, these systems have failed to: (1) optimize conditions for chondrogenic maturation, including favorable growth media; (2) attain high rates of differentiation to the cartilage cell lineage; (3) progress through stages of chondrogenic maturation, ultimately to individual chondrocytes; and (4) exclude lineage determination to related mesengenic pathways.

Summary of the Invention

The present invention is based at least, in part, on the discovery that pleuripotent mesenchymal cells can be cultured *in vitro*, in the presence of specified glycosaminoglycans, such that chondrogenesis is achieved. Further development of the cultures results in the formation of implantable cartilaginous cultures for use in repairing cartilage injury or degeneration as well as reconstructing cartilage *in vivo*. In particular, it has been discovered that the glycosaminoglycan, hyaluronic acid, is sufficient to promote expression of the chondrogenic phenotype and that chondrocytes so-generated are capable of being grown into implantable cultures.

Accordingly, the present invention relates to methods for promoting chondrogenesis and/or making cartilage *in vitro*. More specifically, the invention relates to methods for promoting chondrogenesis in culture, which includes proliferating (*e.g.*, expanding) a population of pleuripotent mesenchymal cells in the presence of a glycosaminoglycan until chondrocytic differentiation is promoted. In one embodiment, the population of pleuripotent mesenchymal stem cells ("PMCs") is cultured until the cells become contact inhibited. In another embodiment, the cells are cultured until at least one chondrocytic or cartilage marker is exhibited. In yet another embodiment, the invention features a method of producing cartilage *in vitro* which includes culturing a population of CD44-positive cells (*e.g.*, PMCs) under conditions such that cartilage is produced.

The present invention further features methods of promoting chondrocytic differentiation (*e.g.*, of a PMC) and methods of promoting chondrogenesis which include treating cells with a chondrogenesis-promoting agent (*e.g.*, a glycosaminoglycan) such that differentiation or chondrogenesis are promoted or enhanced.

The present invention also features implantable cartilaginous cultures as well as methods of producing such cultures. In one embodiment, the methods include culturing PMCs in the presence of a chondrogenesis-promoting agent (*e.g.*, a glycosaminoglycan) such that chondrogenesis is promoted and/or cartilage is produced. Preferred glycosaminoglycans include but are not limited to chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid (*e.g.*, low molecular weight hyaluronic acid).

The methods of the invention are useful in the reconstruction of cartilage, and/or methods of repairing bone-related injury, methods of repairing cartilage injury and methods of promoting normal bone growth. Moreover, the methods of the present invention can be used to replace or augment existing cartilage tissue and to join together biological tissues or structures.

Other features and advantages of the invention will be apparent from the following detailed description, examples and claims.

Detailed Description of the Invention

The present invention relates to methods for promoting chondrogenesis and/or making cartilage *in vitro*. More specifically, the invention relates to methods for promoting chondrogenesis in culture, which includes proliferating (*e.g.*, expanding) a population of pleuripotent mesenchymal cells in the presence of a glycosaminoglycan until chondrocytic differentiation is promoted. In one embodiment, the population of pleuripotent mesenchymal stem cells ("PMCs") is cultured until the cells become contact inhibited. In another embodiment, the cells are cultured until at least one chondrocytic or cartilage marker is exhibited. In yet another embodiment, the invention features a method of producing cartilage *in vitro* which includes culturing a population of CD44-positive cells (*e.g.*, PMCs) under conditions such that cartilage is produced.

The present invention further features methods of promoting chondrocytic differentiation (*e.g.*, of a PMC) and methods of promoting chondrogenesis which include treating cells with a chondrogenesis-promoting agent (*e.g.*, a glycosaminoglycan) such that differentiation or chondrogenesis are promoted or enhanced.

The present invention also features implantable cartilaginous cultures as well as methods of producing such cultures. In one embodiment, the methods include culturing PMCs in the presence of a chondrogenesis-promoting agent (*e.g.*, a glycosaminoglycan) such that chondrogenesis is promoted and/or cartilage is produced. Glycosaminoglycans include but are not limited to chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid (*e.g.*, low molecular weight hyaluronic acid). A preferred glycosaminoglycan is hyaluronic acid (*e.g.*, low molecular weight hyaluronic acid). Preferably, production of cartilage is evidenced by the detection of at least one chondrocytic marker or cartilage marker. Chondrocytic markers (or cartilage markers) include but are not limited to chondroitin-4-sulfate, chondroitin-6-sulfate, a chondrocyte-specific sulfated proteoglycan, type II collagen and aggrecan. Production of cartilage can further be evidenced microscopically and/or ultrastructurally. Most preferably, the cartilage produced by the herein-described methodologies is hyaline cartilage.

The methods of the invention are useful in the reconstruction of cartilage, and/or methods of repairing bone-related injury, methods of repairing cartilage injury and methods of promoting normal bone growth. Moreover, the methods of the present invention can be used to replace or augment existing cartilage tissue and to join together biological tissues or structures. Accordingly, in one embodiment the present invention features a method of repairing cartilage *in vivo* which includes producing cartilage *in vitro* according to one of the herein-described methodologies and implanting the cartilage in a patient at a site in need of augmentation. In another embodiment, the invention features a method of augmenting cartilage *in vivo* which includes producing cartilage *in vitro* according to one of the herein-described methodologies and implanting the cartilage in a patient at a site in need of augmentation.

For convenience, certain terms employed in the specification, examples and claims are collected here.

The term "pleuripotent mesenchymal cell" ("PMC") is defined as a progenitor cell of mesenchymal origin which is not yet committed to adopt a specific lineage. A "pleuripotent mesenchymal cell" or "PMC" is also recognized in the art as a "pleuripotent stem cell" or "mesenchymal stem cell". A PMC is further defined by its ability to undergo an unlimited number of mitotic cell divisions in order to propagate its own phenotype. Alternatively, a PMC has the potential to generate progeny which are referred to herein as "lineage-committed" or "lineage-immature" cells, *i.e.*, cells which are incapable of undergoing multiple mitotic cell divisions and which will eventually differentiate into a specific cell type (*e.g.*, myocytes, osteoblasts, adipocytes, chondrocytes, or fibroblasts).

The term "cartilage" is art-recognized and generally refers to a dense connective tissue containing cells embedded in an extracelllular matrix. Cartilage consists primarily of fibrils or type II collagen in the form of arcade-like structures that are distended by the presence of highly-charged proteoglycans. Naturally occurring cartilage (*i.e.*, cartilage existing in an animal, for example, a human, or alternatively isolated form an animal) can also be characterized by the presence of, at least, type IX collagen, type X collagen, type XI collagen, proteoglycans and hyaluronic acid or hyaluronate. Naturally occurring cartilage contains about 10% cells (*e.g.*, chondrocytes) and about 90% extracellular matrix (Hammerman and Schubert, 1962) comprising mainly type II collagen fibers, proteoglycan macromolecules and water. Water constitutes about 75% of cartilage (depending on the type of cartilage) while the collagen fibers constitute about 15-25% of the wet weight of cartilage (or half the dry weight). The proteoglycans are present in variable amounts (Stockwell,1979) and can account for up to 10% of the wet weight of cartilage (or about a quarter of the dry weight).

There are at least three types of cartilage (*e.g.*, naturally occurring cartilage), each differing from the other in their fiber content. "Hyaline" cartilage is sparse in collagen fibers and accordingly, appears bluish-white, glassy, and translucent both macroscopically and microscopically. "Fibrocartilage" has a higher collagen and lower proteoglycan content, therefore its fibers are macroscopically visible. "Elastic cartilage" contains elastic fibers in the extracellular matrix in addition to the collagen and proteoglycans. Hyaline cartilage is well suited to withstand compressive forces and is

found on the articular surfaces of most long bones (*e.g.*, in the joints), while fibrocartilage is found where shearing forces are applied (Ogden *et al.*, 1975). Hyaline cartilage is also found in costal cartilages (*e.g.*, cartilage connecting the ribs and bones), in septum of nose, as well as in the larynx and trachea.

The term "proteoglycan" includes polymers having repeating units made of polysaccharide (about 95%) covalently attached to a polypeptide backbone or "core protein" (about 5%). The polysaccharide portions (also referred to as glycosaminoglycan) are made up of disaccharide repeating units including, but not limited to, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, or hyaluronic acid. Typically, the ground substance of cartilage is proteoglycan.

The terms "hyaluronic acid", "hyaluronate" or "hyaluronan" include polymers which are composed of repeated disaccharide units of hyalobiuronic acid. In cartilage *in vivo* (*e.g.*, naturally-occurring cartilage), hyaluronic acid ("HA") exists as an elongated filament to which the core proteins of proteoglycans are covalently attached. This interaction is promoted by small link proteins *in vivo*. As defined herein, hyaluronic acid or hyaluronate can be purified from *in vivo* sources and thus can exist in an "isolated" form. "Isolated" HA refers to HA which has been purified from tissue sources or from medium of hyaluronic acid-producing cells (*e.g.*, hyaluronic acid-producing bacteria). Alternatively, HA can be chemically synthesized. Accordingly, "isolated" HA is substantially free of contaminating materials (*e.g.*, proteins) from the cell or tissue source from which it is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of contaminating material" includes preparations of HA which are separated from components of the cells or tissue from which it is isolated or bacterially produced. In one embodiment, the language "substantially free of contaminating material" includes preparations of HA having less than about 30% (by weight) of contaminating materials, more preferably less than about 20% of contaminating materials, still more preferably less than about 10% of contaminating materials, and most preferably less than about 5% contaminating materials. When the HA is bacterially produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about

20%, more preferably less than about 10%, and most preferably less than about 5% of the preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of HA which are separated from chemical precursors or other chemicals which are involved in the synthesis of the polymer. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of HA having less than about 30% (by dry weight) of chemical precursors or non-HA chemicals, more preferably less than about 20% chemical precursors or non-HA chemicals, still more preferably less than about 10% chemical precursors or non-HA chemicals, and most preferably less than about 5% chemical precursors or non-HA chemicals.

As defined herein, "chondrogenesis" includes the differentiation of pleuripotent mesenchymal cells, followed by expression of cartilage-specific genes producing extracellular matrix proteins, and maturation of chondroblasts into chondrocytes which occurs concomitant with suppression of alternative phenotypes.

The term "CD44" refers to a plasma membrane glycoprotein which acts as the principal receptor for hyaluronic acid. *In vivo*, CD44 is involved in cell-cell and cell-matrix adhesion and is expressed by hematopoetic cells and by other tissues such as the lung, brain, smooth muscle, connective tissue and proliferating epithelial cells. Its expression is particularly high in osteocytes, osteoclasts and periosteal cells. It is not, however, expressed by osteoblasts (Hughes, 1994). In the developing embryo, CD44 is expressed in the condensing limb bud mesenchyme prior to the cartilage-generating chondrogenic process. Recent evidence indicates that PMC transiently express a specific CD44 isoform on their surface prior to their migration and condensation, termed CD44v.

The term "condensation" includes the controlled aggregation of PMCs in a manner such that chondrogenesis occurs in the absence of cellular hypertrophy. *In vivo*, chondrogenesis occurs early in the development of the cartilaginous skeleton when PMCs migrate from the notochord (a region low in hyaluronic acid) toward strips of hyaluronate-rich lateral plate mesoderm called the Wolffian Ridges, forming regions of high cell density (Hall and Miyale, 1995). Condensation *in vivo* precedes the

differentiation of mesenchymal cells into chondrocytes producing cartilage matrix (Fell, 1925; Searls *et al.*, 1972).

The term "aggregation" includes an accumulation of cells (*e.g.*, PMCs) in an uncontrolled manner such that cells within the aggregate undergo hypertrophy, as compared to differentiation. Aggregation can result, for example, from cells being plated in culture at too high a density or not being plated as a monodisperse suspension. As used herein, "aggregation" is a non-desirable phenomenon by contrast to condensation, which is desirable and necessary for chondrogenesis.

I. Sources and Culturing of Pleuripotent Mesenchymal Stem Cells

The methods of the present invention are based at least in part on the discovery of the fact that chondrogenesis can be induced and cartilage can be synthesized utilizing as a starting material pleuripotent mesenchymal cells ("PMCs"). Accordingly, one aspect of the invention features PMCs isolated from animals, preferably from human, more preferably from donor-matched humans, and most preferably from the patient in need of cartilage reconstruction or repair or in need of a cartilage implant. In one embodiment, PMCs are derived from the periosteum of an animal, preferably a human. The periostum, as defined herein, refers to the fibrous membrane localized at the surface of bones (*e.g.*, ileum, scapula, tibia, fibula, femur, etc.). In one embodiment, periosteal tissue can be excised arthroscopically by removing a portion of, for example, the rib, tibia, or femur of a patient in advance of the surgery scheduled to reconstruct, repair or implant the cartilage cultures. The periostial tissue is harvested and incubated with collagenase in the appropriate medium (*e.g.*, Ham's F12 media) for about 3 hours at about 37°C, preferably with agitation. The tissue is then disrupted by vortexing or other mechanical means, filtered, and washed. Cells are plated at appropriate concentrations.

In another embodiment, PMCs are derived from the skin of an animal, preferably a human. For example, skin can be cleaned with betadine and rinsed several times with sterile PBS. Skin biopsies can be taken (*e.g.*, 1mm^2 biopsies) and the cells dissociated with a collagenase-dispase mixture (*e.g.*, 1 mg/ml collagenase-dispase). Dispase is included to minimize fibrocyte contamination of primary skin cultures.

Tissue samples can then be digested (*e.g.*, at 37°C for 1 h) and cells resuspended at a concentration sufficient for plating (*e.g.*, 1-5 x 10^6 cells/ml).

Yet another aspect of the invention features genetically engineered cells as a source of PMCs. As used herein, the term a "genetically engineered cell" includes a cell which has been genetically manipulated to overexpress a level of gene product greater than that expressed prior to manipulation of the cell or in a comparable cell which has not been manipulated. Genetic manipulation can include, but is not limited to, altering or modifying regulatory sequences or sites associated with expression of a particular gene (*e.g.*, by adding strong promoters, inducible promoters or multiple promoters or by removing regulatory sequences such that expression is constitutive), modifying the chromosomal location of a particular gene, altering nucleic acid sequences adjacent to a particular gene, increasing the copy number of a particular gene, modifying proteins (*e.g.*, regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of a particular gene and/or translation of a particular gene product, or any other conventional means of increasing expression of a particular gene which is routine in the art. The term "overexpressed" or "overexpression" includes expression of a gene product at a level greater than that expressed prior to manipulation of a cell or in a comparable cell which has not been manipulated. In one embodiment, the genetically engineered cell has been genetically manipulated to overexpress CD44 (*e.g.*, a fibroblastic cell overexpressing CD44).

Yet another aspect of the present invention features established cell lines as a source of pleuripotent mesenchymal cells.

Hyaluronic Acid or Hyaluronate

As previously described, hyaluronic acid or hyaluronate for use in the present invention can be added to cultures of PMCs or, alternatively, can be used to coat vessels (*e.g.*, tissue culture dishes) into which PMCs are plated. Accordingly, in one embodiment, HA for use in the present invention is an isolated preparation of HA, or isolated HA. In one example, isolated HA is derived from a tissue source (*e.g.*, from vitreous bodies, for example, vitreous bodies of bovine eyes, from nasal septum, from umbilical cords, from cartilage, for example, cetacean cartilages, from skin or from fowl

crests). In yet another embodiment, isolated HA is derived from a cellular source (*e.g.*, from the culture broth of HA-producing bacteria). In yet another embodiment, HA is synthesized chemically.

A preferred HA for use in the present invention is low molecular weight HA. As used herein, "low molecular weight" HA is HA having a molecular weight of about 5-200 kDa, preferably about 10-100 kDa, more preferably about 20-80 kDa, and even more preferably 30-50 kDa. In is also within the scope of the present invention to use high molecular weight HA. As used herein, "high molecular weight" HA is HA having a molecular weight of greater than 200 kDa, preferably greater than 500 kDa, and more preferably greater than 1000, 1500, 2500 kDa. The use of high molecular weight HA with the methods of the present invention is sufficient to promote chondrogenesis *in vitro*. However, the present inventor has observed that cells (*e.g.*, PMCs) which have been treated with high molecular weight HA may attempt to clear or process the HA from the extracellular environment during the proliferative or expansion phase of generating a cartilage culture. In particular, the cells (*e.g.*, PMCs) may internalize and/or digest high molecular weight HA, resulting in a slowing of proliferation of cells in the culture both *in vitro* as well as *in vivo*, once the cartilage culture as been implanted. Use of high molecular weight HA has also been observed to slow the differentiation of cells (*e.g.*, PMCs). While, in general, the slowing of proliferation of the cells within the implant may be undesirable, one of skill in the art will appreciate that the effect may have the beneficial side effect of slowing the proliferation of potentially invading cells, for example, macrophages. Potentially invading cells, for instance, may be detrimental to cartilaginous implants used in joint-specific treatments (*e.g.*, the treatment of arthritis).

II. Growth of Cartilage Cultures *in vitro*

The present invention features implantable cartilage cultures for use in the treatment of a wide variety of cartilage disorders *(e.g.*, degenerative disorders, genetic disorders) and for use in a variety of reconstructive and/or augmentative procedures. Accordingly, the pleuripotent mesenchymal cells as described above are cultured *in vitro* under conditions such than chondrogenesis is promoted (*i.e.*, the chondrocytic phenotype

is enhanced) and/or cartilage is produced. In one embodiment, the methods of the present invention include a step involving proliferation or expansion of the pleuripotent mesenchymal cells. In a preferred embodiment, the proliferation or expansion step is carried out in the presence of hyaluronic acid. Growing, culturing or expanding the PMCs in the presence of hyaluronic acid includes, but is not limited to, culturing the cells in the presence of soluble HA and/or culturing the cells on plates coated with HA. In a preferred embodiment, isolated HA as described above can be diluted to a concentration of about 10 μg/ml -100 mg/ml soluble HA with an aqueous solution (*e.g.*, diluted with buffer, for example carbonate buffer or phosphate-buffered saline) and added to dishes for a period of time sufficient to achieve deposition of the HA from the solution onto the surface of the dish. In one embodiment, soluble HA is added to the dishes at a concentration of about 10 μg/ml to100 μg/ml, preferably at a concentration of about 100 μg/ml to 1 mg/ml, more preferably at a concentration of about 1 mg/ml to 10 mg/ml, even more preferably at a concentration of about 10 mg/ml to 100 mg/ml. It has been observed that when using high molecular weight HA, the lower concentrations of soluble HA may be preferable, for example, from about 10 μg/ml to 10 mg/ml.

In one embodiment, the soluble HA is added to the dishes and incubated for at least about 1-2 hours, preferably about 2-4 hours, more preferably about 4-16 hours, and even more preferably about 16-24 hours. In another embodiment, the soluble HA is incubated on the dishes at about 4°C, preferably at about 25°C, and more preferably about 37°C. In yet another embodiment, the solution can be aspirated from the HA-coated dishes and the dishes can be dried (*e.g.*, dried in a laminar flow hood in a sterile environment). In another embodiment, the solution can be aspirated and the dishes can be washed with media or buffer prior to plating PMCs on the coated dishes.

It is likewise within the scope of the present invention to culture, grow or expand cells (*e.g.*, PMCs) in the presence of soluble HA, as compared to culturing, growing or expanding the cells (*e.g.*, PMCs) on HA-coated dishes. In an exemplary embodiment, isolated HA as described above can be diluted to a concentration of about 10 μg/ml -100 mg/ml soluble HA with an aqueous solution (*e.g.*, diluted with media) and the cells can be suspended in the HA-containing media prior to plating on tissue culture dishes. In

another embodiment, the soluble HA is made at a concentration about 10 to 100X that desired in the final media and added to cells which have been plated in non-HA containing media. In yet another embodiment, the HA-containing media is replaced at least about every 72 hours and preferably about every 48 or 24 hours.

It is also within the scope of the present invention to grow, culture or expand cells (*e.g.*, PMCs) on HA-coated dishes further in the presence of HA-containing media. As described above, alternative preferred embodiments include growing, culturing or expanding cells in the presence of chondroitin sulfate (*e.g.*, chondriotin-4 sulfate or chondroitin-6 sulfate), dermatan sulfate or keratan sulfate.

The methods of the present invention further provide for expanding cells (*e.g.*, PMCs) until the cells cease to proliferate due to contact inhibition. In particular, contact inhibition is desirable such that the differentiated chondrocytic phenotype may be achieved. It is also preferable to plate cells at a density such that proliferation and differentiation proceed at a controlled pace. For example, cells (*e.g.*, PMCs) plated at too low a cell density are slow to reach adequate cell numbers for forming cartilage cultures or proliferate and/or differentiate in localized populations with vast spaces of tissue culture dish surface remaining between populations. Alternatively, plating cells at too high a cell density results in uncontrolled aggregation, rather that the desired condensation. Accordingly, in a preferred embodiment, cells are plated at a density of about 1000 to 30,000 cells/cm^2, more preferably about 2000 to 25,000 cells/cm^2, even more preferably about 5000 to 20,000 cells/cm^2, and even more preferably about 7500 to 10,000 cells/cm^2 In an exemplary embodiment, cells are suspended at about 25,000 to 250,000 cells/ml and 1 ml cells are plated per well in a 6-well tissue culture dish (9.6 cm^2) (see for example, Example 7). For generating smaller cartilage cultures, cells can be plated in 12-well dishes (~3.8 cm^2), 24-well dishes (~2.0 cm^2), 48-well dishes (~0.75 cm^2), or 96-well dishes (~0.32 cm^2). For larger cartilage cultures, cells can be plated in 35mm dishes (~9.6 cm^2), 60mm dishes (~19-21 cm^2), 100mm dishes (~58 cm^2), or 150mm dishes (~152 cm^2). Plating cells at the above-described densities is favorable to promote condensation and subsequent chondrocytic differentiation and generation of cartilage. In a preferred embodiment, cells (*e.g.*, PMCs) are plated at a density sufficient to achieve confluence within one week. Further culture of confluent cells results in the

deposition of matrix. In a preferred embodiment, cultures containing cells and cartilage result from an addition.

Because the cultures are to be used for implantation *in vivo*, it preferable to obtain the PMCs from the patient's own tissue (*e.g.*, skin or periosteum) or from the tissue of a biologically related subject or, in the alternative, form a fetal source. Moreover, the implantable culture may further comprise one or more other components, including, for example, selected antibiotics, anesthetics, anti-inflammatory compounds to reduce the risk of rejection if the implant, or immunosuppressants. The growth of implantable cartilaginous cultures may be further enhanced by including other bioactive materials (*e.g.*, growth factors including, but not limited to, TGF-βs, BMPs (*e.g.*, BMP-2, BMP-12, BMP-13) or IGFs). The growth of implantable cartilaginous cultures can further be supplemented by the addition of extracellular matrix components. For example, during limb-bud development prior to chondrogenesis, the extracellular matrix surrounding the mesenchymal cells is primarily composed of type I and type III collagen, fibronectin and proteoglycans (von der Mark *et al.*, 1976; Dessau *et al.*, 1980; Silver *et al.*, 1981; Kulyk *et al.*, 1989b; Shinomura *et al.*, 1990). Additionally, matrix molecules which are components of basement membrane, such as laminin and type IV collagen, are found throughout the developing limb bud, including the regions in which condensation and chondrogenesis occur (Solursh and Jensen, 1988). Accordingly, preferred additional matrix components which can be included in growing, implantable, cartilatagenous cultures include, but are not limited to type I, type III collagen, fibronectin, laminin and/or type IV collagen.

In another embodiment, cytochalasin D can be added to PMCs to enhance the production of cartilage cultures. Cytochalasin D is a microfilament-disrupting agent which can cause cells to become round. Cytochalasin D has been shown to modify the cytoskeleton of dedifferentiated chondrocytes, causing them to round up and this change in the cytoskeleton also stimulated chondrogenesis (Loty, 1995).

The cartilage cultures can be implanted by a number of means routinely practiced in the art. For example, the cultures can be implanted surgically into patients (*e.g.*, arthroscopic surgery). Preferred patients are human patients although the methods of the present invention are equally useful to other mammals in need of repair or augmentation

including, but not limited to horses, dogs, sheep, pigs, and/or cattle. An advantage of the present invention is in the reconstruction of many structures including the nose, nasal septum, ear, respiratory system or parts of the rib cage. Cartilage repair or augmentation is also advantageous in the joins of patients, for example, when cartilage is damaged by inflammation, trauma, or aging, and to repair cartilage which is congenitally defective. A particularly advantageous use of the implantable cartilage cultures of the present invention is the instance where a bone is fractured or broken (*e.g.*, a femoral or tibial fracture), particularly one of the long bones of a child. For example, in instances where the fracture or breakage occurs at the end of the bone, *i.e*, in the proximity of the growth plate, it may be desirable to implant a cartilaginous culture onto the end of the bone such that bone repair processes may proceed in a controlled fashion (*e.g.*, bone repair may take place absent the formation of a fibrous callous such that the child's bone may continue to grow).

III. Determination of the Chondrogenic Phenotype

In vivo, chondrogenesis is characterized by the transition from pluripotent mesenchymal stem cell to committed chondroprogenitor cell (*i.e.*, mesenchymal cells which are committed to the chondrocytic differentiation pathway yet are undifferentiated, *i.e.*, "lineage-committed cells"), to chondroblast, to chondrocyte, and these cells can be distinguished from one another based primarily on their relative maturity (Hall, 1983). The various stages can be differentiated, for example, by the presence or expression of various markers or marker proteins.

Moreover, chondrocytes are characterized by the extracellular matrix that they secrete, *i.e.*, chondroitin-4- and chondroitin-6- sulfates are produced in high quantities by chondrocytes, and their presence has served as a useful criterion in studies of chondrogenic differentiation (Levitt and Dorfman, 1973; Kosher and Lash, 1975; Kosher *et al.*, 1979a,b; Kosher and Savage, 1980). Accordingly, in one embodiment, a marker of the chondrocytic phenotype or "cartilage marker" includes chondroitin-4- or chondroitin-6- sulfate and determination of said chondrocytic marker or "cartilage marker" includes determination of the presence of and/or levels of chondroitin-4- and/or chondroitin-6- sulfate produced by the cells of a culture (*e.g.*, as determined by detection

of chondroitin-4- and/or chondroitin-6- sulfate synthesis, detection of glycosaminoglycan levels, and/or detection of glycosaminoglycan secretion, for example, detection of the presence or levels of the glycosaminoglycan in the medium of the culture).

Furthermore, experimental evidence has shown that there are differences between the kinds of sulfated proteoglycans produced by chondrocytes, precartilagenous mesenchymal cells, and by nonchondrogenic cell types. It has been demonstrated that proteoglycans synthesized by embryonic chick chondrocytes can be separated into two major fractions by molecular sieve chromatography on either agarose (Palmoski and Goetinck, 1972; Levitt and Dorfman, 1974) or controlled-pore glass beads (Lever and Goetinck, 1976). The larger of these fractions makes up about 90% of the total proteoglycan produced by embryonic chondrocytes and consists of aggregated and unaggregated proteoglycan monomers (Goetinck *et al.*, 1974; Lever and Goetinck, 1976). Synthesis of this larger proteoglycan fraction by sternal and limb chondrocytes is selectively inhibited by 5-bromodeoxyuridine (Palmoski and Goetinck, 1976; Levitt and Dorfman, 1974), and is drastically reduced in chondrocytes of the cartilage-defective nanomelic mutant chick embryo (Palmoski and Goetinck, 1972; Pennypacker and Goetinck, 1976a; McKeown and Goetnick, 1979). Thus it has been suggested that the larger proteoglycan fraction represents a cartilage-specific species and the smaller fraction, a ubiquitous nonspecific species (Palmoski and Goetinck, 1972; Levitt and Dorfman, 1973, 1974). Sucrose density gradient has also been used to demonstrate that embryonic chondrocytes synthesize a large proteoglycan species produced neither by precartilagenous mesenchymal cells nor by a variety of nonchondrogenic cell types (Okayama *et al.*, 1976; Kitamura and Yamagata, 1976).

More compelling evidence supporting the existence of a cartilage-specific proteoglycan molecule is derived from immunologic studies. Antibodies prepared against the large proteoglycan monomers of 4-week old chick sternal cartilage do not cross-react with the proteoglycans produced by precartilaginous limb mesenchymal cells (Royal *et al.*, 1980) or chick skin (Sparks *et al.*, 1980). The antibodies do, however, cross-react with proteoglycans produced by embryonic chick and quail sternal cartilage, chick Meckel's cartilage and chick limb cartilage (Sparks *et al.*, 1980; Royal *et al.*,

1980). Similarly, antibodies prepared against epiphyseal cartilage proteoglycan monomer from which most of the chondroitin sulfate glycosaminoglycan chains have been removed by hyaluronidase treatment, show little, if any, cross-reactivity with the proteoglycans of precartilaginous limb mesenchymal cells (Ho *et al.*, 1977). Furthermore, immunohistochemical studies show that limb mesenchymal cells that have differentiated into cartilage in high-density monolayer culture, react with antibodies to hyaluronidase-digested cartilage proteoglycan, while noncartilage cells in the same culture do not (Vertel and Dorfman, 1978). These studies indicate that the proteoglycan molecule which is produced by chondrocytes compared to the proteoglycan molecule produced by precartilaginous mesenchymal cells or nonchondrogenic cell types, is unique, and may differ in its protein core.

Accordingly, in another embodiment, a marker of the chondrocytic phenotype or "cartilage marker" includes chondrocyte-specific sulfated proteoglycans and determination of said chondrocytic marker or "cartilage marker" includes determination of the presence of and/or levels of said chondrocyte-specific sulfated proteoglycan produced by the cells of a culture (*e.g.*, as determined by detection of chondrocyte-specific sulfated proteoglycan synthesis, detection of chondrocyte-specific sulfated proteoglycan levels, *e.g.*, using antibodies, molecular sieve chromatography and/or density gradient ultracentrifugation), and/or detection of chondrocyte-specific sulfated proteoglycan secretion, for example, detection of the presence or levels of the chondrocyte-specific sulfated proteoglycan in the medium of the culture).

A second major macromolecule in the extracellular matrix of cartilage is collagen. The predominant collagenous component of cartilage matrix is type II collagen (Miller and Matukas, 1969; Miller, 1976), although small amounts of other collagen species including type V may be present (Rhodes and Miller, 1978; Burgeson and Hollister, 1979; Shellswell *et al.*, 1980). Type II collagen is a triple helical molecule consisting of three identical polypeptide chains (called alpha1 (II) chains). The chains are distinct gene products differing in primary structure from the alpha chains of the other known collagens. It is found in the extracellular matrix of hyaline cartilages from a variety of sources and is absent from tissues such as bone, tendon and skin (Miller, 1976).

By contrast, precartilaginous mesenchymal cells produce type I collagen (Linsenmayer *et al.*, 1973a; von der Mark *et al.*, 1976; von der Mark and von der Mark, 1977; Bailey *et al.*, 1979; Dessau *et al.*, 1980). *In vivo* and *in vitro* studies have shown that type II collagen is not produced by myogenic and connective tissue cells that differentiate in association with limb cartilage (Linsenmayer *et al.*, 1973a; von der Mark *et al.*, 1976; von der Mark and von der Mark, 1977; Bailey *et al.*, 1979; Dessau *et al.*, 1980). Type II collagen is also not produced by limb mesenchymal cells whose differentiation into cartilage *in vitro* has been inhibited by 5-bromodeoxyuridine (Levitt and Dorfman, 1974) or by low-density culture conditions (von der Mark and von der Mark, 1977). Accordingly, in another embodiment, a marker of the chondrocytic phenotype or "cartilage marker" includes type II collagen and determination of said chondrocytic marker or "cartilage marker" includes determination of the presence of and/or levels of type II collagen produced by the cells of a culture (*e.g.*, as determined by detection of type II collagen synthesis synthesis, detection of type II collagen levels, *e.g.*, using Northern blot analysis, western blot analysis, ELISA and the like), and/or detection of type II collagen, for example, detection of the presence or levels of type II collagen in the medium of the culture or matrix of the cells of the culture).

Some nonchondrogenic tissues are capable of producing type II collagen and because of this it cannot be described as being a cartilage-specific molecule. It is produced by the embryonic notochord (Linsenmayer *et al.*, 1973b; von der Mark *et al.*, 1976; Miller and Matthews, 1974), embryonic chick corneal epithelium (Linsenmayer *et al.*, von der Mark *et al.*, 1977), and neural retina (Newsome *et al.*, 1976; Smith *et al.*, 1976; Linsenmayer and Little, 1978). Type II collagen can however, be considered to be cartilage-characteristic because it is produced by only a limited number of nonchondorgenic cell types and it is not produced by precartilaginous mesenchymal cells or the fibroblastic and myogenic cells that differentiate in association with cartilage. Accordingly, it is within the scope of the present invention to include chondrocyte-specific markers as well as chondrocyte-characteristic markers within the meaning of the phrase "chondrocytic marker" or "cartilage marker".

In yet another embodiment, the presence of aggrecan can be a marker of chondrocytic differentiation or cartilage marker.

IV. Determination of the Presence of Hyaline Cartilage

Ultrastructurally, hyaline cartilage matrix is characterized by numerous densely staining granules and thin unbanded or faintly banded fibrils representing the proteoglycan and collagenous components of the matrix (Matukas et al, 1967; Anderson and Sadjera, 1971; Searls *et al.*, Minor, 1973; Levitt *et al.*, 1975; Pennypacker and Goetinck, 1976). Under the light microscope, hyaline cartilage appears homogeneous and structureless, and is characterized by its ability to stain metachromatically in toluidine blue, or positively in Alcian blue. These staining patterns reflect the high concentration of polyionic sulfated glycosaminoglycans present in the matrix.

At a cellular level, differentiated chondrocytes are typically rounded or polygonal cells with scalloped borders. Their cytoplasm contains an extensive network of rough endoplasmic reticulum and large Golgi complexes and secretory vacuoles, suggesting that the cells are actively synthesizing and secreting extracellular matrix components (Godman and Porter, 1960; Goel, 1970; Searls *et al.*, 1972; Thorogood and Hinchliffe, 1975).

Accordingly, any of the above-described visual, histologic and/or microscopic characteristics can be utilized as markers of the chondrocytic phenotype or "cartilage markers".

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, and published patent applications) cited throughout this application are hereby expressly incorporated by reference.

Exemplification

EXAMPLE 1: INDUCTION OF CHONDROGENESIS IN PLEURIPOTENT MESENCHYMAL STEM CELLS

Isolation of Primary Pleuripotent Mesenchymal Cells, Stimulation of Condensation and Initiation of Differentiation

This Example describes the isolation of primary skin and periosteal cells and demonstrates the stimulation of condensation with hyaluronic acid and initiation of differentiation. This example also demonstrates that hyaluronic acid-treated cells express CD44(v3) and co-expressed integrin β3 subunit.

Isolation of Primary Skin Cells and Periosteal Cells

Mice skin was cleaned with betadine and rinsed several times with sterile PBS. $1mm^2$ skin biopsies were taken and the cells were dissociated with 1 mg/ml collagenase-dispase mixture. Dispase is included to minimize fibrocyte contamination of primary skin cultures. Tissue samples were digested at 37°C for 1 h and single cells were resuspended at a concentration of 5×10^6 cells/ml. Periosteal cells were isolated according to art-recognized procedures.

Characterization of Hyaluronic Acid-Treated Cells

100μl aliquots of cells were placed in 96-well plates plated on either hyaluronate (HA)-coated dishes (10 mg/ml high molecular weight HA or 100 mg/ml low molecular weight HA) or plates on plastic tissue culture dishes. Hyaluronate induced the aggregation of periosteal cells (CD44 positive, see below) within 1 hour after plating, whereas cells cultured on uncoated dishes remained as single cells.

One week after plating, cells were fixed and stained with either Toluidine Blue or Safranine O/Fast Green for visualization. Again, hyaluronate-induced cell aggregation was apparent *(e.g.,* two weeks after plating).

Immunocytochemical Straining for CD44

Mouse periosteal cells were cultured for 14 days on either HA-coated or uncoated culture dishes. Cells were fixed with 1% paraformaldehyde, 1% gluteraldehyde in 0.1 M cacodylic acid (pH 7.3). CD44 was visualized by indirect immunofluorescent microscopy using Rhodamine optics under 200 X magnification.

Immunocytochemical Straining for β_3

Periosteal cells or skin cells were cultured for two weeks either HA-coated or uncoated culture dishes. Cells were fixed and stained for β_3 subunit of integrin. Original magnification was 200 X.

Table 1:

	CD44(all)*	β3	Mac 1	total
Skin	78	1	6	100
Periosteal	67	3	1	100

*CD44 refers to all CD44. < 1% of the population contains exon3. CD44v3 β3 cells represent less than 0.5% of total skin cells.

EXAMPLE 2: INDUCTION OF CHONDROGENESIS IN CD44-EXPRESSING FIBROBLASTS

CD44-Positive Cells Undergo Ligand-Induced Aggregation

This Example demonstrates that, in the presence of appropriate ligand, cells that are CD44 positive undergo ligand-induced aggregation mediated by the interaction of several CD44 receptors on multiple cells with a single multivalent glycosaminoglycan (GAG) molecule. The binding of GAGs to CD44 induces the capping of the CD44 receptor. The capping of the CD44 receptor induces cell to cell binding mediated by as yet unidentified receptors which induces cell condensation. This process mimics what is observed *in vivo* during limb bud development. To analyze the consequences of cell

condensation and to determine whether this phenomena is a prerequisite for chondrogenesis, cell condensation as mediated by several GAGs was analyzed and the developmental fate of the condensates was examined.

GAG-Induced Condensation of a CD44 Negative Cell Line (A31 Control Cell Line) Aggregation Assay: Only aggregates of 4 or more cells are considered positive for aggregation. Only the wells which display at least 50% of the cells in aggregate are considered to score positive for aggregation.

A31 cells were investigated for the production of aggregate. 1 x 10^5 A31 cells were incubated in calcium- and magnesium-free PBS with 5mg/ml low molecular weight hyaluronate (HAL) or high molecular weight hyaluronate (HAH) or 5mg/ml chondroitin sulfate C (CS) for 5 h at room temperature then incubated at 37°C in media up to 24 hours. At the 0 minute interval, all of the A31 control cells were isolated but they maintained an aggregation of 5% between the 0 minute and 5 hour period. At the 1 hour interval, 5% of the A31 cells which were exposed to high and low molecular weight HA and chondroitin sulfate C formed aggregates. Between 3 and 5 hours, the A31 cells consisted of 10% of its cells in aggregates when low and high molecular weight HA and chondroitin sulfate C were added. Cultures of A31 cells without any ligand did not aggregate. Since none of the fields showed more than 50% of cells in aggregates, it was concluded that GAGs did not induce cell aggregation of A31 cells.

Table 2:

Ligand	% cells in aggregates
Control	5±5
Hyaluronic acid (Low MW)	10±5
Hyaluronic acid (High MW)	10±5
Chondroitin sulfate	10±5

GAG-induced Condensation of a CD44-Positive Cell Line

CD44-positive cells (1 x 10^5 cells) were incubated in calcium- and magnesium-free PBS with 5mg/ml low molecular weight hyaluronate (HAL) or high molecular weight hyaluronate (HAH) or 5mg/ml chondroitin sulfate C (CS) as described above. Approximately 200 cells per well were counted for each group and were scored as positive when more than 50% of the cells were involved in aggregates of four or more cells. At the 0 minute interval, 10% of the $CD44^+$ cells had aggregated. 60% of the $CD44^+$ cells formed aggregates after 1 hour in low or high molecular weight HA or in chondroitin sulfate C. Between 3 and 5 hours, 85% of the $CD44^+$ cells, which were exposed to low molecular weight hyaluronic acid formed aggregates. Of the cells which were exposed to high molecular weight hyaluronic acid or chondroitin sulfate C, 70% scored positive for aggregation. Cultures of $CD44^+$ cells without any ligand did not aggregate. Since more than 50% of the cells per field where found in aggregates of 4 or more, it was concluded that GAGs induced the aggregation of $CD44^+$ cells.

Table 3:

Ligand	% cells in aggregates
Control	10 ± 5
Hyaluronic acid (Low MW)	$85^*\pm10$
Hyaluronic acid (High MW)	$70^*\pm10$
Chondroitin sulfate	$70^*\pm10$

* $p<0.01$

These results suggest that multiple cells can bind to a single molecule of the multivalent GAGs. This interaction mediates cell-to-cell interaction which results in cell condensation. In the absence of glycosaminoglycans (GAGs), no cell aggregation is observed suggesting that this phenomena is due to GAGs and not simply the result of cell clumping. These results also suggest that the A31 cells which lack CD44 cannot aggregate or condense in response to GAGs.

EXAMPLE 3: CHARACTERIZATION AND/OR DETERMINATION OF THE CHONDROGENIC PHENOTYPE

CD44-Positive Cells Display Phenotypic Characteristics of Differentiated Chondrocytes

This example demonstrates that CD44-positive, but not control cells, display markers and shape characteristic of the differentiated chondrocyte phenotype.

Immunocytochemical Staining for CD44 of CD44-Positive and A31 cells which are exposed to Hyaluronate or Chondroitin Sulfate C

After 24 hours of incubation, 80% of the $CD44^+$ cells which were exposed to high or low molecular weight hyaluronic acid or to chondroitin sulfate C were round and stained positively for CD44. The $CD44^+$ cells which were not exposed to the ligands (control) appeared flat and polygonal in shape, and 80% stained positively at the cell surface. The $CD44^+$ cells which were exposed to chondroitin sulfate C were flat and polygonal and 70% of the cells stained positively at the cell surface.

The A31 cells which were exposed to high or low molecular weight hyaluronic acid or to chondroitin sulfate C and the control cells appeared flat and polygonal and stained negative for CD44.

These findings confirm that the $CD44^+$ cells remained CD44-positive throughout the aggregation and condensation period and possess CD44 cell surface receptors while the A31 cells do not express CD44 cell surface receptors and remained CD44-negative.

Immunocytochemical Staining for Type II Collagen of CD44-Positive and A31 cells which are Exposed to Hyaluronate or Chondroitin Sulfate C.

After one week of incubation, 80% of the $CD44^+$ cells which were exposed to high or low molecular weight hyaluronic acid, expressed positive staining for collagen type II with highest expression in the periphery of the aggregates. 40% of the $CD44^+$ 10T1/2 cells which were exposed to chondroitin sulfate C stained positively for type II collagen. The cells which were not exposed to the ligands of the control did not express type II collagen. The A31 cells, which were incubated with HAL, HAH, CS or the control did not exhibit positive staining for type II collagen.

These findings indicate that the $CD44^+$ cells, in the presence of high or low molecular weight hyaluronic acid or chondroitin sulfate C, differentiated into cells which expressed a chondrogenic phenotype. The A31 cells exhibited no staining for type II collagen after one week, indicating that the A31 cells did not progress to cells which expressed a chondrogenic phenotype.

Immunocytochemical Staining for Type I Collagen of CD44-Positive and A31 cells which are Exposed to Hyaluronate or Chondroitin Sulfate C.

After one day and after one week, none of the $CD44^+$ cells stained positively for type I collagen. The A31 cells also did not stain positively for type I collagen. These results suggest that the $CD44^+$ aggregates did not differentiate into cells which expressed an osteogenic phenotype. The A31 cells also did not produce cells which express an osteogenic phenotype.

Histochemical Staining with Safronin O of CD44-Positive and A31 Cells which are Exposed to Hyaluronate or Chondroitin Sulfate C.

After one day, the A31 cells which were exposed to high or low molecular weight hyaluronic or to chondroitin sulfate C and the control (no ligand) were flat and polygonal and stained intracellularly with safronin O but there was no extracellular staining, suggesting that the cells did not synthesize or secrete new proteoglycans into the surrounding extracellular matrix. After one week the A31 cells demonstrated the same pattern of staining as the one day group.

The aggregated $CD44^+$ 10T1/2 cells which were incubated with high or low molecular weight hyaluronic acid or with chondroitin sulfate C stained positively after one day. The control $CD44^+$ cells (no ligand) also exhibited positive intracellular staining after one day. This suggests that the cells did not synthesize or secrete new proteoglycans into the surrounding extracellular matrix.

After one week, the $CD44^+$ cells which were exposed to high or low molecular weight hyaluronic acid, were round in shape. These cells stained positively with more intense staining around the aggregates. The control cells (no ligand) and the cells exposed to chondroitin sulfate C were also round and stained positively on the periphery

of the aggregates. This positive staining suggests that the differentiated cells synthesized proteoglycans which were secreted into the surrounding extracellular matrix.

EXAMPLE 4: Mechanism of Action of Hyaluronate-Induced Chondrocyte Differentiation

This Example describes the mechanism by which CD44 aggregation induces markers characteristic of the chondrogenic phenotype.

Induction of Collagen II(a1) Gene and the Activation of ERK-1 and ERK-2 by Hyaluronic Acid.

Previous studies have demonstrated that ligation of the CD44 receptor with HA results in the rapid activation of the small G-protein p21. Activated G-proteins can induce Map kinase which subsequently can activate cFOS, a transcription factor necessary for the activation of chondrocytic genes. To examine the role of CD44/HA in the activation of chondrocytic genes and to begin investigating the signal transduction pathway responsible for the activation of chondrocytic genes, collagen II(α1) gene expression and Map kinase activity were examined in an A31 embryonic mesynchymal cell line, stably transfected with CD44. The cell line, A31.C1, expresses CD44(v3-v7) on its cells surface. These cells, but not the mock transfected cell line A31.MLV, have been shown to condense in the presence of hyaluronic acid.

1 x 10^6 A31.C1 or A31.mlv cells were treated with 1 mg/ml PBS and incubated at 4°C. After 1 h, the media was removed and replaced with DME containing 10% FBS. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2. After 24h, RNA was extracted from the cultures and Northern blot analysis performed. A31.c1 cells up regulate colII expression in response to hyaluronic acid (HA), however neither the mock transfected cells, A31.MLV, nor the untreated A31.C1 express significant levels of colII (α1).

To test for potential signaling pathways involved in activation of colII(α1) gene, CD44-positive A31.C1 cells were examined for Map kinase activation. Treatment of A31.C1 with hyaluronic acid results in rapid activation of erk-1 and erk-2 as judged by immunoprecipitation of ^{32}P labeled erk-1 and erk-2. However, neither untreated cells

nor mock transfected cells activate Map kinase in response to HA. These results present evidence that the activation of colII(α1) may be mediated through MAP-kinase.

Taken together, these data demonstrate that when CD44 negative PMC are transfected with CD44, MAP kinase is activated and turns on the ColIIα1 gene which expresses type II collagen. Other experiments demonstrated that when CD44 is transfected into other cells, the expression of ColIIa1 is not induced, suggesting that PMC are encoded with genes for, and are therefore primed for chondrocytic differentiation.

EXAMPLE 5: Enhancement of the Chondrogenic Phenotype by Activation of Sox 9 and/or Mutation of Chondrocyte-Specific Silencers

Sox9 is a member of a family of genes which encodes transcription factors with a high mobility group (HMG)-type DNA binding domain. Heterozygous mutations in Sox9 in humans leads to campomelic dysplasia (CD), a severe dwarfism syndrome characterized by anomalies in all skeletal elements derived from cartilages (Forster *et al.*, 1994).

Sox9 is expressed in mesenchymal cell condensations in the embryo before and during cartilage deposition, but its expression ceases after the cartilage matrix is laid down. This short period of expression suggests that Sox9 has a role during initiation and maintenance of chondrogenesis and is no longer required once chondrogenesis is complete. When chondrocytes undergo dedifferentiation in culture, the expression of Sox9 is lost, indicating that the expression of Sox9 is necessary for the maintenance of the chondrocytic phenotype. Whole mount *in situ* hybridization studies in mouse embryos have shown that Sox9 is highly expressed during gonadal development as well as during chondrogenesis (Wright, 1995).

Sox-9 is a gene which mediates the expression of ColIIa1 (the gene which expresses type II collagen) by activating chondrocyte-specific enhancers on the ColIIa1 gene (Bell, 1997; Lefebvre, 1997; Zhao, 1997). Sox9 inactivates the CIIS1 and CIIS2 silencers of aggrecan, a gene essential in chondrogenic progression. Moreover, the c-fos gene is expressed by PMC in the developing embryo only after they begin to undergo condensation and it plays a role in the transition of mesenchymal cells to the

chondrogenic phenotype (Edwall-Arvidsson and Wroblewski, 1996). It can be hypothesized that the CD44/HA bond releases intracellular calcium when capped CD44 binds to ankyrin, causing disruption of the cytoskeleton. MAP kinase becomes phosphorylated and it activates the c-fos gene. The Sox-9 gene is also activated but by an alternate pathway and the ColIIa1 gene is expressed.

In embryology, the migrating PMC already express Sox-9, condensation activates the c-fos gene and together both genes upregulate the ColIIa1 gene which is expressed in chondrocyte-like cells. Likewise, the cells Example 1 are derived from embryonic mesenchyme and may already be encoded to express the Sox-9 gene. By contrast, it may be desirable to induce Sox9 expression in cells such as those used in Examples 2-3 or, alternatively, to mutate silencers of chondrocytic genes (*e.g.*, CIIS1 and/or CIIS2 in aggrecan. Induction of Sox9 expression can be achieved, for example, by transiently or stably expressing the gene in mammalian cells or by contacting cells with a compound which induced expression. Mutation of chondrocytic silencers can be achieved, for example, by mutating a base within one or both of CIIS1 and/or CIIS2.

The following conclusions can be readily drawn from the above examples: (1) the interaction between CD44 and hyaluronate (HA) is necessary but not sufficient for pleuripotent mesenchymal cells (PMC) to condense and then differentiate into chondrocyte-like cells; (2) condensation is mediated by low molecular weight HA or chondroitin sulfate C but not by high molecular weight HA; (3) chondroitin sulfate C mediates the formation of larger aggregates while low molecular weight HA mediates more aggregates with less cells per aggregate.

Moreover, this investigation demonstrates the effect of CD44 on pleuripotent mesenchymal cells (PMC) in the presence of various glycosaminoglycans. It illustrates that the CD44/HA interaction induces PMC to undergo condensation and then differentiation into cells which express a chondrogenic phenotype. It also shows that the presence of CD44 is necessary but not sufficient for the differentiation of these PMC. This study also presents a model to explain the signal transduction pathway which mediates the differentiation of PMC into chondrocyte-like cells. The pathway begins with CD44 and ends by expression of the chondrogenic phenotype with the activation of the ColIIa1 gene.

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

What is claimed:

1. A method of producing an implantable cartilaginous culture comprising culturing pleuripotent mesenchymal cells (PMCs) in the presence of a chondrogenesis-promoting agent such that an implantable cartilaginous culture is produced.

2. The method of claim 1, wherein said chondrogenesis-promoting agent is a glycosaminoglycan.

3. The method of claim 2, wherein said glycosaminoglycan is selected from the group consisting of chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid.

4. The method of claim 3, wherein said agent is hyaluronic acid.

5. The method of claim 3, wherein said agent is low molecular weight hyaluronic acid.

6. The method of claim 1, wherein production of cartilage is evidenced by the detection of at least one cartilage marker.

7. The method of claim 1, wherein said cartilage is hyaline cartilage.

8. A method of promoting chondrocytic differentiation of a pleuripotent mesenchymal cell comprising treating a cell with a chondrogenesis-promoting agent such that chondrocytic differentiation is promoted.

9. A method of promoting chondrogenesis comprising contacting a cell with a chondrogenesis-promoting agent such that chondrogenesis is enhanced.

10. The method of claim 8 or 9, wherein said cell is a pleuripotent mesenchymal stem cell.

11. The method of claim 7 or 8, wherein said chondrogenesis-promoting agent is a glycosaminoglycan.

12. The method of claim 11, wherein said agent is a low molecular weight glycosaminoglycan.

13. The method of claim 8, wherein chondrocytic differentiation is evidenced by at least one indicator of chondrocytic differentiation.

14. The method of claim 13, wherein said indicator of chondrocytic differentiation is selected from the group consisting of chondroitin-4-sulfate, chondroitin-6-sulfate, a chondrocyte-specific sulfated proteoglycan, type II collagen and aggrecan.

15. A method of producing cartilage *in vitro* comprising:

(a) proliferating a population of pleuripotent mesenchymal cells in the presence of a glycosaminoglycan until the cells of said population become contact-inhibited; and

(b) culturing said population until at least one cartilage marker is exhibited.

16. A method of repairing cartilage *in vivo* comprising producing cartilage *in vitro* according to the method of claim 15 and implanting said cartilage in a patient at a site in need of repair.

17. A method of augmenting cartilage *in vivo* comprising producing cartilage *in vitro* according to the method of claim 15 and implanting said cartilage in a patient at a site in need of augmentation.

18. A method of producing cartilage *in vitro* comprising culturing a population of CD44-positive cells under conditions such that cartilage is produced.

19. The method of claim 18, wherein said CD44-positive cell is a pleuripotent mesenchymal cell.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/31607

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 35/00; C12P 21/00; C12N 15/85
US CL :424/93.1, 93.21; 435/70.1; 435/325, 354, 357
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.21; 435/70.1; 435/325, 354, 357

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG, DERWENT WPI, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,919,702 A (PURCHIO et al) 06 July 1999, col. 1, line 1-col. 3, line 38, col. 6, line 43-59, col. 10, line 43-col. 21, line 42.	1-6, 8-13, 15-17 --- 7, 14, 18, 19
X --- Y	SOLCHAGA, L.A. et al. Hyaluronic Acid-Based Polymers as Cell Carriers for Tissue-Engineered Repair of Bone and Cartilage. Journal of Orthopaedic Research. March 1999, Vol 17, pages 205-213, see entire article.	1-6, 8-13, 15-17 --- 7, 14, 18, 19
X --- Y	GIRDLER, N.M. In Vitro Synthesis and Characterization of a Cartilaginous Meniscus Grown from Isolated Temporomandibular Chondroprogenitor Cells. Scand. J. Rheumatol. 1998, Vol. 27, pages 446-453, see entire article.	1-13, 15-17 --- 14, 18, 19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P"	document published prior to the international filing date but later than the priority date claimed		

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